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Detection of intermediary Clr with complete active site, using a synthetic proteinase inhibitor

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The synthetic proteinase inhibitor, FUT-175 (6-amidino-2-naphthyl-4-guanidinobenzoate), strongly suppressed activation of Clr at 37°C, causing 50% inhibition at 0.03 mM. To clarify whether the inhibitor was incorporated into the active site of intermediary Clr formed during the incubation, determination of the active site was tried using this inhibitor. Consequently, release of amidinonaphthol equimolar with the amount of Clr used was observed in the early period of incubation, in which the activation to $\overline{Clr}$ was about 5%. These results indicate that intermediary Clr already has a complete active site.

Synthetic proteinase inhibitor *6-Amidino-2-naphthyl-4-guanidinobenzoate* *FUT-175* *Clr activation* *Intermediary Clr* *Clr active site determination*

1. INTRODUCTION

The first component of complement, Cl, consists of 3 subcomponents, Clq, Clr and Cls, which form a complex in the presence of Ca^{2+} [1]. When Cl binds to the immune complex via Clq, Clr proenzyme is converted into an active proteinase, which catalyzes the proteolytic activation of Cls [2]. The most interesting problem in the Cl activation mechanism is how the Clr proenzyme is activated upon binding of Clq to the immune complex [3–6].

Although there are some inconsistencies in interpretation of the activation mechanism, the activation of Clr has been generally accepted to be an autocatalytic reaction [7–14]. Furthermore, several workers speculated that the proteolytic site for activation of Clr is generated in the Clr proenzyme through a conformational change [8,9,15]. Recently, direct proof of the conformational change of Clr during the activation process at 37°C was obtained using a hydrophobic fluorescent probe [16]. However, the relation between conformational change of Clr and active site formation is still not clear.

Recently, we developed a new synthetic proteinase inhibitor, FUT-175 [17]. This inhibitor strongly inhibits almost all serine proteinases involved in coagulation, kallikrein–kinin, fibrinolysis and complement systems. To clarify the interesting problem of the relation between the conformational change and active site formation, we tested the effect of this inhibitor on activation of Clr, and examined whether the possible active site formed with a conformational change of Clr could be determined using the inhibitor.

2. MATERIALS AND METHODS

The following reagents were obtained commercially: leupeptin (Protein Research Foundation,

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Abbreviations: FUT-175, 6-amidino-2-naphthyl-4-guanidinobenzoate; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; DFP, diisopropyl fluorophosphate; SDS–PAGE, SDS-polyacrylamide gel electrophoresis

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Japan), DFP (Katayama, Japan), NPGB–HCl (Sigma, MO), Sepharose 6B and CM-Sephadex C-50 (Pharmacia, Sweden), BioGel A-1.5 m (Bio-Rad, USA). Human fresh frozen plasma was obtained from the Japan Red Cross. FUT-175 and acetylglycyl-L-lysine α-naphthyl ester were prepared in the Research Laboratories of Torii & Co., Japan.

2.1. *Complement component*

Clr was isolated as in [6] with extensive modifications. A sample of 1000 ml human serum containing 1 mM DFP, 100 mg soybean trypsin inhibitor and 3 mM benzamidine was diluted with 4 l cold water containing the above inhibitor, allowed to stand overnight at 4°C, and then centrifuged. The small precipitate of euglobulin was partially dissolved by addition of 15 ml of 50 mM acetate buffer (pH 5.5), containing 200 mM NaCl, 5 mM $CaCl_2$ and 1 mM DFP, and centrifuged for 60 min at 105000 × g. The supernatant was fractionated on a column (5 × 90 cm) of Sepharose 6B in the same buffer. Active fractions were pooled, treated with FDP at a final concentration of 1 mM, concentrated to 100 ml by ultrafiltration and dialyzed against 50 mM acetate buffer (pH 5.5), containing 50 mM NaCl and 5 mM EDTA. The dialyzate was applied to a CM–Sephadex C-50 column (2.5 × 10 cm) equilibrated with the same buffer and material was eluted with a linear gradient of 50–500 mM NaCl in 2000 ml starting buffer. Active fractions were pooled, concentrated and applied to BioGel A-1.5 m in 50 mM acetate buffer (pH 5.5) containing 500 mM NaCl and 5 mM EDTA. About 4 mg purified Clr was obtained by these procedures and stored at −70°C before use.

For detection of Clr, 10–20 μl of each fraction was incubated with 700 μl of 100 mM phosphate buffer (pH 7.0), and 100 μl of 0.01% trypsin solution in the same buffer for 5 min at 37°C. Then the trypsin was neutralized by addition of 100 μl of 0.5% soybean trypsin inhibitor solution in the same buffer. The esterase activity of Clr after trypsin treatment was determined with 0.2 μmol acetylglycyl-L-lysine α-naphthyl ester as substrate [18]. The purified Clr proenzyme gave a single protein band with an apparent M_r of 88000 on SDS–PAGE in the reduced form, while after incubation at 37°C heavy and light chains with apparent M_r values of 55000 and 35000 appeared under reducing conditions.

2.2. *SDS–PAGE*

Electrophoresis was done in 7% polyacrylamide gel under reducing conditions as in [19]. Samples of 8.8 μg Clr in 125 mM Tris buffer (pH 7.4) containing 1.5 mM $CaCl_2$ and 150 mM NaCl were incubated for 60 min at 37°C with or without various inhibitors in a total volume of 40 μl. Then the samples were heated for 5 min at 80°C with 1% SDS and 2% 2-mercaptoethanol and analyzed by SDS–PAGE. The extent of Clr activation was monitored densitometrically (Toyo digital densitorol DMU-33C) after staining the protein with Coomassie brilliant blue.

2.3. *Fluorescence measurement*

The active site was determined by measuring the fluorescence of amidinonaphthol released in the enzyme reaction, using FUT-175. A mixture of 100 μl Clr (0.57 nmol) in 125 mM Tris buffer (pH 7.4) containing 1.5 mM $CaCl_2$ and 150 mM NaCl (ice-cold), 380 μl of the same buffer (prewarmed to 37°C) and 20 μl FUT-175 (57 nmol) in distilled water (ice-cold) was rapidly mixed in a cell and promptly incubated at 37°C. Release of amidinonaphthol was monitored in a Hitachi fluorescence spectrophotometer, model 650-10M, using excitation and emission wavelengths of 330 and 480 nm, respectively. The control mixture was the same, but without enzyme. The fluorescence of amidinonaphthol released with enzyme minus that released without enzyme was defined as the amount of active site. Under these conditions, the fluorescence intensity of amidinonaphthol was stable and no fluorescence of FUT-175 itself was observed. The extent of activation of Clr without FUT-175 under the same conditions was analyzed by SDS–PAGE as described above.

2.4. *Protein determination*

Clr was measured from its absorbance at 280 nm using $E^{1\%}_{1cm}$ = 11.2 [12].

3. RESULTS

3.1. *Effect of FUT-175 on activation of Clr at 37°C*

The inhibitory effects of FUT-175 and other in-

hibitors on the activation of Clr at 37°C were investigated, and results are illustrated in fig.1. The most potent inhibitor was FUT-175 which, at 0.03 mM, caused 50% inhibition. Although NPGB was also strongly inhibitory, its effect was 1/5 that of FUT-175 (0.15 mM). DFP and leupeptin were active at higher concentrations (3.1 and 1.7 mM, respectively). These results suggest that these inhibitors may be incorporated into Clr proenzyme, and consequently suppress the activation to $\overline{Clr}$. The differences in the effects of these inhibitors may result from differences in their accessibilities to the active site. To investigate whether the active site is actually formed in Clr proenzyme and whether the inhibitor is incorporated into the active site, we tried to determine the active site formed in Clr proenzyme using FUT-175, which was the most effective inhibitor of activation of Clr.

3.2. *Determination of the active site of Clr proenzyme using FUT-175*

Fig.2 shows the amount of amidinonaphthol released from FUT-175 in the enzyme reaction of Clr proenzyme during incubation at 37°C. As can be seen, the release of amidinonaphthol gradually increased during incubation at 37°C, and after about 10 min amidinonaphthol was released in an amount equimolar to that of Clr used. On the other hand, the rate of activation to $\overline{Clr}$ after 10 min incubation in the absence of FUT-175 was about 5%. These results strongly suggest that the proenzyme Clr (single chain) with a complete active site appeared in the lag phase during the early period of activation.

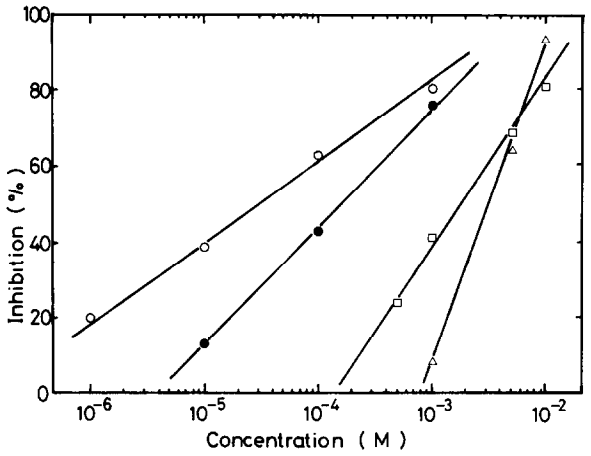

Fig.1. Effects of various inhibitors on Clr activation. Clr (220 μg/ml) was incubated in 125 mM Tris buffer (pH 7.4) containing 1.5 mM $CaCl_2$ and 150 mM NaCl for 60 min at 37°C, in the presence of FUT-175 (○), NPGB (●), DFP (△) or leupeptin (□) at final concentrations of 0.001–1 mM (FUT-175 and NPGB) or 0.1–10 mM (DFP and leupeptin). After reduction, samples were analyzed by SDS–PAGE, and the amount of $\overline{Clr}$ was determined by scanning the gels after staining with Coomassie blue.

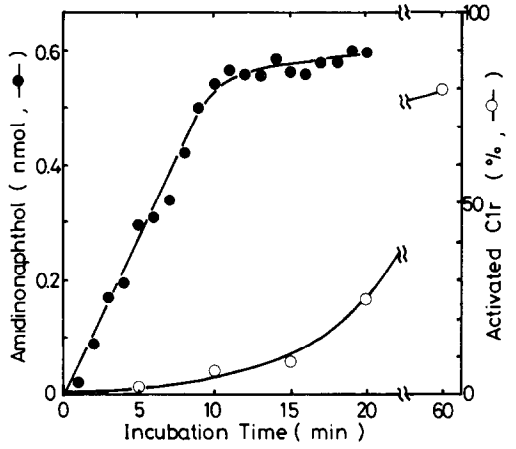

Fig.2. Determination of the active site of Clr. A mixture of Clr (0.57 nmol) and FUT-175 (57 nmol) was incubated in 500 μl 125 mM Tris buffer, containing 1.5 mM $CaCl_2$ and 150 mM NaCl. The fluorescence of amidinonaphthol released was monitored in a Hitachi fluorescence spectrophotometer using excitation and emission wavelengths of 330 and 480 nm, respectively. The extent of activation to $\overline{Clr}$ in the absence of FUT-175 was analyzed by SDS–PAGE, as described for fig.1.

4. DISCUSSION

FUT-175 is a synthetic inhibitor of trypsin-like serine proteinases [17]. A special feature of this inhibitor is that it strongly inhibits proteinases involved in the complement system, such as $\overline{Clr}$, $\overline{Cls}$, B and $\overline{D}$, as well as proteinases involved in coagulation, kallikrein–kinin and fibrinolysis systems [20]. Its inhibition mechanism is supposed to be similar to that of NPGB, since FUT-175 is also a derivative of guanidinobenzoate. However, the inhibition of $\overline{Clr}$ by FUT-175 was about 4-times that by NPGB [17,21]. The reason for this

is unknown, but possibly the amidinonaphthol moiety of FUT-175 participates in the inhibition. Here, we investigated the effect of FUT-175 on the activation of Clr. Results indicated that FUT-175 was about 5-times more effective than NPGB. This result is consistent with their relative inhibitions of $\overline{Clr}$.

In inhibition of the activation of Clr, NPGB has been reported to interact with intermediary Clr (Clr*) formed during the activation process, and so its inhibition of the activation to $\overline{Clr}$ is reversible [8]. The active site in Clr* is supposed to be generated by a conformational change occurring in the early phase of activation, but is no positive evidence that formation of the active site involves a conformational change of Clr proenzyme, and the incorporation of an inhibitor into the active site formed has not been proposed. Accordingly, proof of the above hypothesis requires determination of the amount of active site formed in Clr*.

When FUT-175 is used as inhibitor, the release of amidinonaphthol with the acylation of an active serine residue by the guanidinobenzoate moiety can be observed. Since amidinonaphthol emits strong fluorescence, by measuring the fluorescence of amidinonaphthol formed by the reaction between FUT-175 and Clr*, the amount of active site formed can be determined. Results indicated that release of amidinonaphthol was equimolar with the amount of Clr used in the early period (about 10 min) of incubation, in which the activation to $\overline{Clr}$ was slight (about 5%). This strongly suggests that Clr* already has a complete active site. Recently, experiments with a hydrophobic fluorescent probe showed that, although Clr has a single chain, its conformation changes rapidly during the early period of incubation [16]. These results can be explained by supposing that the rapid conformational change of Clr in the early period of incubation causes formation of the complete active site, and that interaction between this active site and the inhibitor suppresses further activation to $\overline{Clr}$.

Recently it was reported that activation of Clr proceeded by a two-step reaction: first an intramolecular catalytic process catalyzed by Clr proenzyme itself, and then an intermolecular reaction catalyzed by $\overline{Clr}$ [14]. This mechanism may well explain the kinetics of autoactivation of Clr, although there are several reports suggesting that $\overline{Clr}$ alone cannot activate Clr [8,9]. Our finding that an intermediary Clr (Clr*) already has a complete active site supports the idea of a two-step reaction in autoactivation of Clr. However, it is not clear whether the conversion of Clr* to $\overline{Clr}$ in the first step proceeds by an intramolecular reaction catalyzed by Clr* itself or by an intermolecular reaction.

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